P444 0001
GOSO/GSL

Paper No.:____

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

Inventor(s):	HUANG, Dong; QI, Dong Feng		
Title:	NOVEL AGLYCON DAMMARANE SAPOGENINS, THEIR USE AS ANTI-CANCER AGENTS, AND A PROCESS FOR PRODUCING SAME		
Serial No.:	09/910887		
Filed:	24 July 2001		
Examiner:	Sabiha Naim Qazi	Art Unit:	1616
Date:	2 December 2002		

Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

AFFIDAVIT UNDER RULE 1.132

I, F. Geoffrey Herring, of 7922 Cartier Street, Vancouver, British Columbia, Canada, V6P 4T4, MAKE OATH AND SAY AS FOLLOWS:

1. I have personal knowledge of the matters sworn to herein, except where the matters are stated to be based on information and belief, in which case I believe them to be true.

2. I received a Bachelor of Science from the University of London in 1961. I also received a Ph.D. in Chemistry from the University of London in 1964.

3. I have worked as a Professor in the Department of Chemistry at the University of British Columbia, in Vancouver, British Columbia, Canada, since 1967. I have consulted for many companies, including pharmaceutical companies, in the area of biochemistry and physics since 1994.

4. I have read United States patent application serial number 09/910,887 (the "Application") and I am familiar with the art to which it pertains.

5. I have read and reviewed the Office Action for the Application, issued on 9 September 2002 by the United States Patent and Trademark Office.

6. I have also read and reviewed Japanese Patent Abstract No. 08291194A2 (the "Japanese Abstract") and PCT Application No. PCT/KR96/00123 (the "PCT Application"), which were cited in the Office Action against the Application. Copies of the Japanese Abstract and the PCT Application are attached as Exhibits "A" and "B" to this affidavit, respectively.

7. The Japanese Abstract discloses two compounds, dammara-20(22),24-diene-3β,12β-diol ("J1") and dammara-20(22),24-diene-3β,6α,12β-triol ("J2").

8. I have compared J1 to the compound dammara-20(21)-diene-3,12-diol ("PAM-120") disclosed in the Application. I find that J1 differs from PAM-120 in the position of the double bond located at position 20. In J1, the double bond is located between positions 20 and 22, and in PAM-120, the double bond is located between positions 20 and 21. In my opinion, the difference between the positions of the double bonds is extremely significant from a chemical activity point of view. A double bond is a planar bond and does not rotate. A double bond between positions 20 and 22 prevents the side chain at position 22 from rotating in J1. However, in PAM-120, the double bond is located between positions 20 and 21, and there is only a single bond between positions 20 and 22. Therefore, the side chain at position 22 is able to rotate in PAM-120. PAM-120 and J1 are not inter-convertible conformations. The difference in the position of the double bond can affect the stereochemistry of reactions between the side chain of PAM-120 and an anti-cancer drug, or a biological molecule, or a biological structure, or all of these. The enhanced conformational flexibility of PAM-120 provides for a wider range of chemical and physico-chemical interactions. Therefore, the chemical and biological activity of PAM-120 cannot be predicted based upon the chemical and biological activity of J1.

9. I have compared J2 to the compound dammara-20(22E)-diene-3,12,24-triol ("PBM-100") disclosed in the Application. I find that J2 differs from PBM-100 in both the composition and position of the side chain extending from carbon 22. More significantly, PBM-100 has an additional hydroxyl group at position 24 and lacks a double bond between positions 24 and 25. In my opinion, the addition of the hydroxyl group at position 24 is extremely significant from a chemical activity point of view because hydroxyl groups are known to be reactive groups and in general

have different chemical activities than aliphatic chains. The presence of the hydroxyl group on the side chain provides for further polar interactions with target molecul s, which in turn could lead to different biological activities with PBM-100 as compared to J2. In addition, the difference in the position of the side chain at position 22 can affect the stereochemistry of the reaction between PBM-100 and an anti-cancer drug, or a biological molecule, or a biological structure, or all of these. Therefore, the chemical and biological activity of PBM-100 cannot be predicted based upon the chemical and biological activity of J2.

10. I have compared J2 to the compound dammara-20(22E)-diene-3,6,12-triol ("PBM-110") disclosed in the Application. I find that J2 differs from PBM-110 in the orientation of the side chain at carbon 22. In my opinion, this difference is extremely significant from a chemical activity point of view because, with respect to the side chain, PBM-110 and J2 are geometrical isomers. PBM-110 is the trans (E) isomer and J2 is the cis (Z) isomer. The disruptive effect of the position of a side chain is unpredictable, especially in molecules with large molecular skeletons. Cis and trans isomers may have very different activities. The orientation of the side chain can affect the stereochemistry of the reaction between PBM-110 and an anti-cancer drug, or a biological molecule, or a biological structure, or all of these. Therefore, the chemical and biological activity of PBM-110 cannot be predicted based upon the chemical and biological activity of J2.

11. I have compared J1 to compounds PBM-100 and PBM1110, and I find that J1 is different because PBM-100 and PBM-110 have additional hydroxyl groups at position 6. In my opinion, this difference is extremely significant from a chemical activity point of view because hydroxyl groups are known to be reactive groups and therefore can affect the reaction between PBM-100 and PBM-110 and an anti-cancer drug, or a biological molecule, or a biological structure, or all of these. Therefore, the chemical and biological activity of PBM-100 and PBM-110 cannot be predicted based upon the chemical and biological activity of J1.

12. I have compared J2 to compound PAM-120, and I find that J2 is different because J2 has an additional hydroxyl group at position 6. In my opinion, this difference is extremely significant from a chemical activity point of view because hydroxyl groups are known to be reactive groups. Because PAM-120 does not have a hydroxyl group at position 6, this may affect the reaction between PAM-120 and an anti-cancer drug, or a biological molecule, or a biological structure, or all of these.

Therefore, the chemical and biological activity of PAM-120 cannot be predicted based upon the chemical and biological activity of J2.

13. I have compared J1 and J2 to the compounds 3-O-β-D-glucopyranosyl-dammara-20(21)-diene-3,12-diol ("PAN-20") and 3-O-[-D-glucopyranosyl(1 2)- -D-glucopyranosyl]-dammara-20(22E)-diene-3,12-diol ("PAN-30"), disclosed in the Application. I find that J1 and J2 are significantly different from PAN-20 and PAN-30 because PAN-20 and PAN-30 have sugar moieties at position 3. In my opinion, this difference is extremely significant from a chemical activity point of view because the sugar moieties make PAN-20 and PAN-30 larger in size as compared to J1 and J2. As such, PAN-20 and PAN-30 may physically interact differently with other molecules. The sugar moieties are themselves reactive and therefore, their presence will affect the biological and chemical activities of PAN-20 and PAN-30. The presence of the sugar moieties provides for additional chemical and physico-chemical interactions which can lead to different biological and chemical activities. Therefore, it would not be possible to predict the activity of PAN-20 and PAN-30 based on the activity of J1 and J2.

14. The PCT Application discloses four compounds, two of which were identified in the Office Action as being significant. The first compound is 3β,12β-dihydroxy-damar-20(22),24-diene-3-O-β-D-6"-O-acetyl-glucopyranosyl-(1-2)-β-D-glucopyranoside ("P1"), which is derived by acetylating the second compound, Δ20(22)-ginsenoside R_{t3} ("P2").

15. I have compared compounds P1 and P2 with compounds PAM-120, PBM-100, and PBM-110. I find that P1 and P2 are significantly different from PAM-120, PBM-100, and PBM-110 because P1 and P2 have sugar moieties at position 3. In my opinion, this difference is extremely significant from a chemical activity point of view because the sugar moieties make P1 and P2 larger in size compared to PAM-120, PBM-100, and PBM-110, and as such P1 and P2 may physically interact differently with other molecules. The sugar moieties are themselves reactive and therefore, their presence will affect the biological and chemical activities of P1 and P2. The presence of the sugar moieties provides for additional chemical and physico-chemical interactions which can lead to different biological and chemical activities. Therefore, it would not be possible to predict the activity of PAM-120, PBM-100, and PBM-110 based on the activity of P1 and P2.

16. I have also compared compound P1 with PAN-20, and I find that P1 differs from PAN-20 because PAN-20 only contains one glucosyl group in its sugar moiety at position 3, whereas P1 contains two glucosyl groups and an acetyl group. In addition, PAN-20 contains a double bond between positions 20 and 21, whereas P1 contains a double bond between positions 20 and 22. In my opinion, these differences between PAN-20 and P1 are extremely significant from a chemical activity point of view because the sugar moiety is a different length and P1 also contains an acetyl group, which is known to have different chemical properties than a glucosyl group. Also, the position of the double bond is significant. As stated above, a double bond is a planar bond and does not rotate. A double bond between positions 20 and 22 prevents the side chain at position 22 from rotating in P1. In PAN-20, the double bond is located between positions 20 and 21, and there is only a single bond between positions 20 and 22. Therefore, in PAN-20, the side chain at position 22 is able to rotate. The difference in the position of the double bond can affect the stereochemistry of reactions between the side chain of PAN-20 and an anti-cancer drug, or a biological molecule, or a biological structure, or all of these. The enhanced conformational flexibility of PAN-20 provides for a wider range of chemical and physico-chemical interactions. Therefore, the chemical and biological activity of PAN-20 cannot be predicted based upon the chemical and biological activity of P1.

17. I have compared compound P1 with PAN-30, and I find that P1 differs from PAN-30 because PAN-30 contains an acetyl group on its sugar moiety, and the orientation of the side chain at position 22 is different. In my opinion, these differences are extremely significant from a chemical activity point of view because acetyl groups are known to have different chemical activities than glucosyl groups. As well, with respect to the side chain, the two compounds are isomers. PAN-30 is the trans (E) isomer and P1 is the cis (Z) isomer. The disruptive effect of the position of a side chain is unpredictable, especially in molecules with large molecular skeletons. Cis and trans isomers may have very different activities. The orientation of the side chain at position 22 can change the stereochemistry of the reaction between PAN-30 and an anti-cancer drug, or a biological molecule, or a biological structure, or all of these. Therefore, the chemical and biological activity of PAN-30 cannot be predicted based upon the chemical and biological activity of P1.

18. I have compared compound P2 with PAN-20, and I find that PAN-20 is different because it only has one glucosyl group in its sugar moiety at position 3 and the position of the double bond at position 20 is between positions 20 and 21, rather than positions 20 and 22 as in P2. In my opinion, these differences are extremely significant from a chemical activity point of view because the sugar moiety is a different length, which makes PAN-20 a smaller molecule than P2 and it may therefore react differently both chemically and physically. As well, the stereochemistry at position 20 is affected by the position of the double bond. As stated above, a double bond is a planar bond and does not rotate. A double bond between positions 20 and 22 would prevent the side chain at position 22 from rotating in P2. However, in PAN-20, the double bond is located between positions 20 and 21, and there is only a single bond between positions 20 and 22. Therefore, the side chain at position 22 is able to rotate in PAN-20. The difference in the position of the double bond can affect the stereochemistry of reactions between the side chain of PAN-20 and an anti-cancer drug, or a biological molecule, or a biological structure, or all of these. The enhanced conformational flexibility of PAN-20 provides for a wider range of chemical and physico-chemical interactions. Therefore, the chemical and biological activity of PAN-20 cannot be predicted based upon the chemical and biological activity of P2.

19. I have compared compound P2 with PAN-30, and I find that P2 differs from PAN-30 in the orientation of the side chain at position 22. In my opinion, this difference is extremely significant from a chemical activity point of view because, with respect to the side chain, PAN-30 and P2 are isomers. PAN-30 is the trans (E) isomer and P2 is the cis (Z) isomer. The disruptive effect of the position of side chains is unpredictable, especially in molecules with large molecular skeletons. Cis and trans isomers may have very different activities. The orientation of the side chain can affect the stereochemistry of the reaction between PAN-30 and an anti-cancer drug, or a biological molecule, or a biological structure, or all of these. Therefore, the chemical and biological activity of PAN-30 cannot be predicted based upon the chemical and biological activity of P2.

20. In addition, a publication entitled "Metabolism of 20(S)- and 20(R)-Ginsenoside R_{g3} by Human Intestinal Bacteria and Its Relation to *in vitro* Biological Activities" by Bae et al., Biol. Pharm. Bull. 25(1) 58-63 (2002), a copy of which is attached hereto as Exhibit "C", indicates that when 20(S)-ginsenoside R_{g3} is incubated with human intestinal microflora, the ginsenoside is hydrolyzed to 20(S)-ginsenoside R_{h2} and 20(S)-protopanaxadiol. However, when 20(R)-ginsenoside R_{g3} is incubated with human intestinal microflora, this ginsenoside is only weakly hydrolyzed. The transformation rate of 20(S)-ginsenoside R_{h2} was 0.57 ± 0.20 nmol/h/mg wet of feces, compared to a rate of 0.03 ± 0.20 nmol/h/mg wet of feces for 20(R)-ginsenoside R_{g3}. Also, it was reported in the publication that 20(S)-ginsenoside R_{h2} and its metabolites have significant *in vitro* cytotoxic affects on tumor cell lines while 20(R)-ginsenoside R_{g3} and its metabolites have little or no cytotoxic effects. 20(R)-ginsenoside R_{g3} and 20(S)-ginsenoside R_{h2} are positional isomers and differ only in their stereochemistry at position 20. The data from this publication indicates that there can be significant differences in biological and chemical activity between positional isomers of ginseng compounds.

SWORN before me at the)
city of Vancouver, in the)
Province of British Columbia,)
Canada this 2nd day of December)
2002.)

A Notary Public in and for the)
Province of British Columbia,)
Canada. My Commission is for life.)

F. Geoffrey Herring

KESHO RAM DITTA
Barrister & Solicitor
1929 West Broadway
Vancouver, B.C. Canada
V6J 1Y5
Ph: (604) 733-8913

O:\WA\GENERAL\BOCHKARYOVA\GSL\P4440001 affidavit.frm

THIS IS EXHIBIT " A " REFERRED TO IN THE

AFFIDAVIT OF F. GEOFFREY HERRING

MADE BEFORE ME THIS

2nd day of December, 2002

A Commissioner for taking Affidavits
or a Notary Public

(19) (11) Publication number: **08291194 A**

Generated Document.

PATENT ABSTRACTS OF JAPAN

(21) Application number: **07115321**

(22) Application date: **18.04.95**

(51) Intl. Cl.: **C07J 9/00** A61K 31/575

(30) Priority:

(43) Date of application publication: **05.11.96**

(84) Designated contracting states:

(71) Applicant: **HAPPY WORLD:KK**

(72) Inventor: **HASEGAWA HIDEO**
SEI SHIYOUKAN
MATSUMIYA TOMOYUKI
UCHIYAMA MASAMORI

(74) Representative:

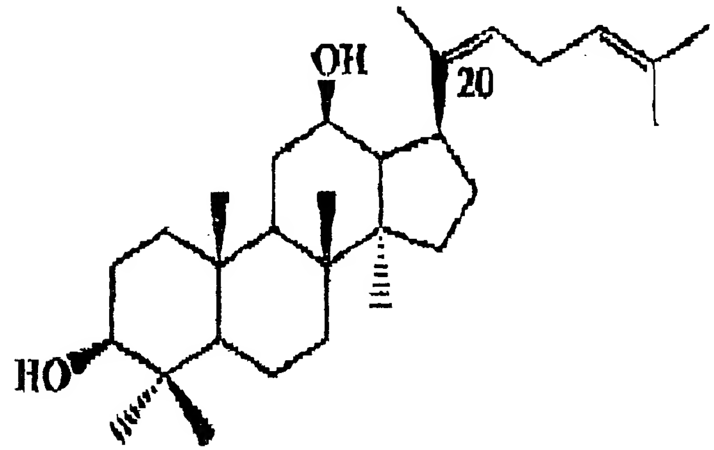
(54) GINSENG SAPOGENIN AND ITS PRODUCTION

(57) Abstract:

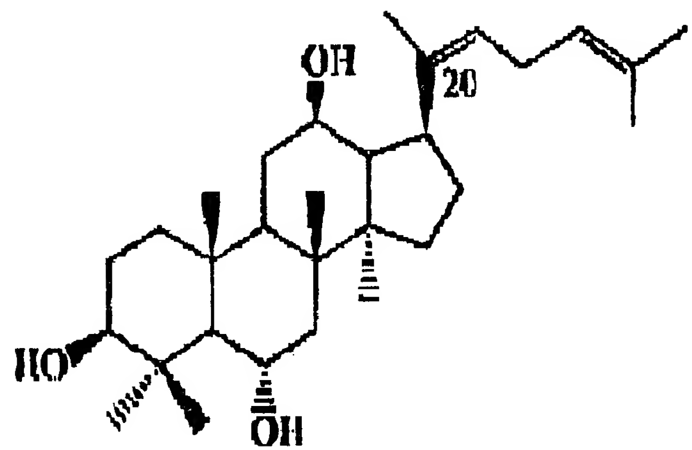
PURPOSE: To obtain a new sapogenin for enhancing agents, etc., capable of increasing sensitivity of cancer to antitumor agent by carrying out hydrolysis and dehydrogenation treatment of glycoside containing 20 (S)-protopanaxadiol and trial extracted from ginseng as a skeleton.

CONSTITUTION: This compound is a new dammara-20(22),24-diene-3β, 12β-diol expressed by formula I or a new dammara-20 (22),24-diene-3β,6α, 12β-triol expressed by formula II. The compound is extremely useful as a enhancing agent capable of remarkably enhancing chemotherapeutic effect of an antitumor agent by increasing sensitivity of a cancer cell which acquired multiple drug resistance to the antitumor agent and

cancelling multiple drug resistance. The compound can be obtained as a sapogenin by carrying out hydrolysis and dehydration treatment of glycoside containing 20 (S)-protopanaxadiol and triol extracted from ginseng (e.g. Panax ginsing) as a skeleton.



I



II

THIS IS EXHIBIT " **B** " REFERRED TO IN THE

AFFIDAVIT OF F. GEOFFREY HERRING

MADE BEFORE ME THIS

2nd day of December, 2002

Keshia Rawn D'iffer

A Commissioner for taking Affidavits
or a Notary Public

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07J 17/00, 75/00, A61K 31/705 A1

(11) International Publication Number: WO 97/31933

(43) International Publication Date: 4 September 1997 (04.09.97)

(21) International Application Number: PCT/KR96/00123

(22) International Filing Date: 29 July 1996 (29.07.96)

(30) Priority Data: 1996/4879 27 February 1996 (27.02.96) KR

(71) Applicant (*for all designated States except US*): CHEIL JE DANG CO. [KR/KR]; 500, Namdaemunro 5-ga, Chung-ku, Seoul 100-095 (KR).

(71)(72) Applicant and Inventor: PARK, Man, Ki [KR/KR]; Ceachi Village #310-1502, 66, Kumi-dong, Bundang-ku, Sungnam-shi, Kyungki-do 463-500 (KR).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): LEE, Seung, Ki [KR/KR]; Hanshin Hankang, Apartment #5-803, 34-13, Banpo-dong, Seocho-ku, Seoul 137-040 (KR). PARK, Jeong, Hill [KR/KR]; Hansol Village, Apartment #301-208, Ilwonbon-dong, Kangnam-ku, Seoul 135-230 (KR). KIM, Jong, Moon [KR/KR]; Chukong 2nd. Apartment #229-313, Chamshil 2-dong, Songpa-ku, Seoul 138-222 (KR). LEE, Kwang, Youl [KR/KR]; 105-1, Jayang-dong, Dong-ku, Daejeon 300-100 (KR). HAN, Sang, Beom [KR/KR]; 1577-24, Pongchun 4-dong, Kwanak-ku, Seoul 151-054 (KR).

(74) Agents: CHOI, Kyu, Pal et al.; 824-20, Yeoksam-dong, Kangnam-ku, Seoul 135-080 (KR).

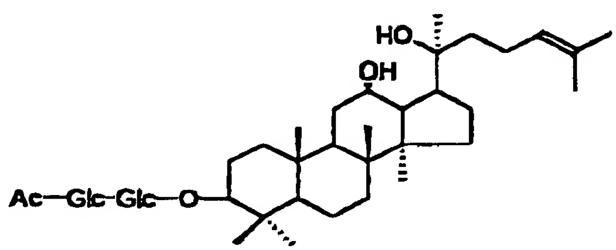
(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

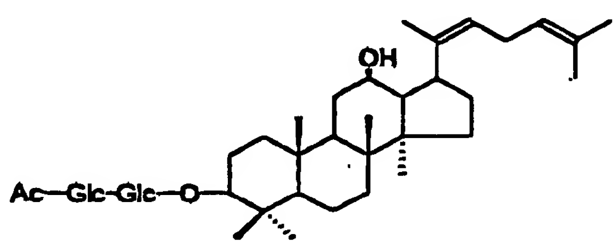
(54) Title: NOVEL GINSENG SAPONIN COMPOUNDS, PROCESS FOR PREPARATION THEREOF AND ANTI-TUMOR AGENT COMPRISING THE SAME AS AN ACTIVE COMPONENT

(57) Abstract

The present invention relates to novel ginseng saponin compounds having a potent anti-tumor activity, which are represented by formulae (I) and (II). The compounds (I) and (II) above are novel and can be produced by heating plants of ginseng genus for 0.5 to 20 hours at a high temperature of 110 to 180 °C, or can be synthesized by acetylating ginsenoside Rg_3 and $\Delta^{20(22)}$-ginsenoside Rg_3 which are known ginseng saponin compounds, respectively. The present invention also relates to an anti-tumor composition comprising these compounds (I) and/or (II) as an active ingredient.



(I)



(II)

FOR THE PURPOSES OF INFORMATION ONLY

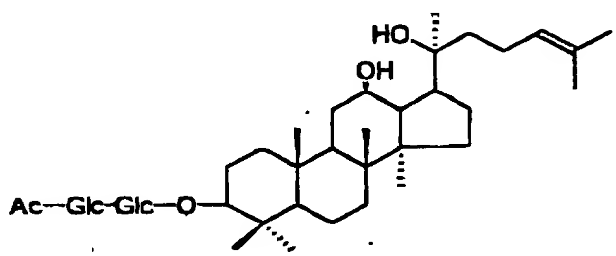
Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgystan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic			SE	Sweden
CG	Congo	KR	Republic of Korea	SG	Singapore
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LR	Liberia	SZ	Swaziland
CS	Czechoslovakia	LT	Lithuania	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	LV	Latvia	TJ	Tajikistan
DK	Denmark	MC	Monaco	TT	Trinidad and Tobago
EE	Estonia	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	UG	Uganda
FI	Finland	ML	Mali	US	United States of America
FR	France	MN	Mongolia	UZ	Uzbekistan
GA	Gabon	MR	Mauritania	VN	Viet Nam

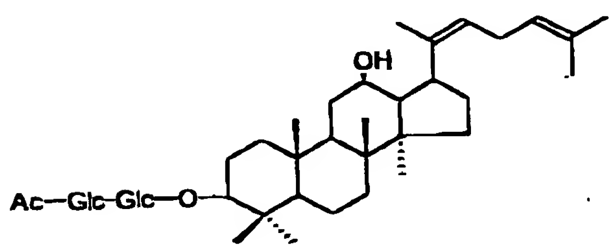
NOVEL GINSENG SAPONIN COMPOUNDS, PROCESS FOR PREPARATION THEREOF AND ANTI-TUMOR AGENT COMPRISING THE SAME AS AN ACTIVE COMPONENT

TECHNICAL FIELD

The present invention relates to novel ginseng saponin compounds having an anti-tumor activity. More specifically, the present invention relates to novel ginseng saponin compounds having a potent anti-tumor activity, which are represented by the following formulas (I) and (II).



(I)



(II)

The present invention also relates to a process for preparation thereof, and an anti-tumor composition comprising the same as an active component.

BACKGROUND ART

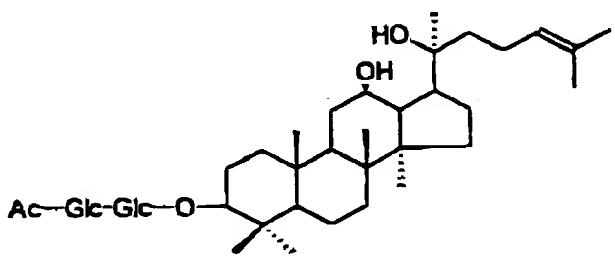
The pysiological and biochemical researches in the effect of ginseng, particularly red ginseng, have been generally conducted on the subject of saponin components which are contained in large quantities in ginseng and has been known as the main component exhibiting the pharmacological effect of ginseng. However, the minor saponin components contained in red ginseng merely in minute quantities have been studied by very few groups heretofore because they can be hardly separated from ginseng.

DISCLOSURE OF INVENTION

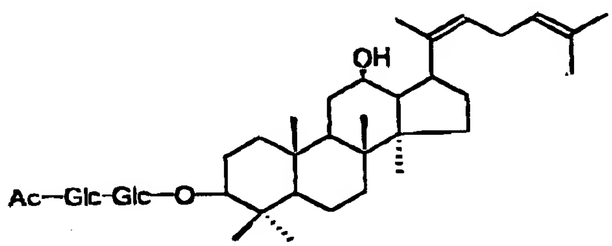
Thus, the present inventors have concentratedly studied to find a method for enhancing the pharmacological effect of ginseng by treating ginseng under specific conditions to increase the contents of specific components and further for separating the respective components so that the study of their pharmacological effect can be made. As a result of such studies, we have identified that when a ginseng is heat-treated for 0.5 to 20 hours at a high temperature of 110 to 180°C, the contents of effective components which are present in a minor amount in ginseng increase, and consequently a processed ginseng having an enhanced pharmacological effect compared with fresh ginseng, white ginseng or red ginseng is prepared. In the procedure to determine the pharmacological effect of the various components separated from the processed ginseng, the present inventors have found novel components which have never been disclosed heretofore, and subsequently identified the chemical structure, pharmacological effect and process for preparation thereof. Thus, we have completed the present invention.

Therefore, the present invention relates to saponin compounds identified as novel active components contained in ginseng.

The first object of the present invention is to provide the novel saponin compounds represented by the following formulas (I) and (II). The configuration at $\Delta^{20(22)}$ of (II) is zusammen or entgegen.



(I)



(II)

It is a further object of the present invention to provide the process for preparing the novel ginseng saponin compounds having the formulas (I) and (II) above.

Further, it is still another object of the present invention to provide an anti-tumor composition comprising the compound (I) and/or (II) as an active component.

BEST MODE FOR CARRYING OUT THE INVENTION

The compounds of formulas (I) and (II) according to the present invention can be prepared by an extraction from the processed product of plant of *Panax* genus or by a synthetic method using known ginsenoside components as a starting material.

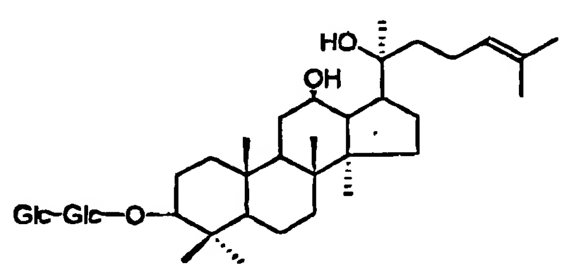
First, according to the extraction method, roots or leaves of the plant of *Panax* genus, for example, *Panax ginseng*, *Panax notoginseng*, *Panax quinquefolium*, *Panax japonicus*, etc., or tissue cultures thereof, or extracts therefrom with water or lower alcohol are heated for 0.5 to 20 hours at a temperature of 110 to 180°C. The processed ginseng thus obtained is extracted with water, or a suitable organic solvent, for example, lower alcohols such as methanol, ethanol, etc., or a solvent mixture thereof, and then the extract is concentrated under reduced pressure, suspended in water and then extracted with a nonpolar organic solvent such as hexane, ether, dichloromethane, chloroform, ethylacetate or a solvent mixture thereof. The remaining aqueous layer is extracted with a polar organic solvent such as butanol and then the extract is subjected to chromatography to obtain a fraction containing compounds (I) and (II). This fraction is crystallized from a suitable solvent system, for example a solvent mixture of water and lower alcohol, preferably a solvent mixture of water and methanol in a ratio of 1:1 by volume, to prepare the desired pure saponin compounds (I) and (II).

According to this method, during the procedure of heat-treatment of ginseng, a sugar moiety attached to the 20th carbon of panaxadiol saponins present in ginseng such as ginsenosides Ra, Rb_1, Rb_2, Rc, Rd, etc. is removed and an acetyl group is introduced into the 6th position of the

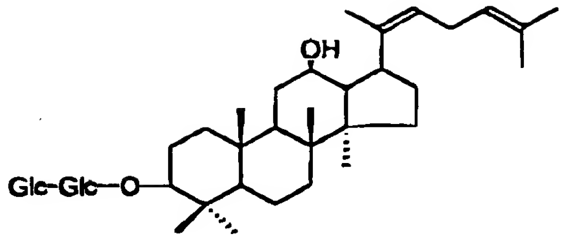
terminal glucose of the sugar moiety attached to 3rd carbon to produce the novel saponin compound (I). The saponin compound (I) can also be produced by removing the sugar moiety attached to the 20th carbon from ginsenosides Rs_1 and Rs_2. The saponin compound (II) is produced by removing the OH group attached to the 20th carbon and hydrogen at the 22th-position from the compound (I) through dehydration reaction. In this reaction, the stereochemical structure of the double bond at 20th-position can have cis or trans configuration.

In the extraction method according to the present invention as mentioned above, the contents of the desired compounds (I) and (II) can be more increased by repeatedly carrying out chromatography. In addition, if necessary, the order of the heat-treatment step and extraction step with organic solvent in this process can be inverted to obtain the same result.

According to the synthetic method of the present invention, the novel saponin compounds of formulas (I) and (II) can be obtained by acetylating the known ginsenoside compounds. Specifically, the compound of formula (I) can be produced by acetylating the known ginsenoside Rg_3 of formula (III), and the compound of formula (II) can be prepared by acetylating the known $\Delta^{20(22)}$-ginsenoside Rg_3 of formula (IV) which is formed by the dehydration reaction at 20th-position of ginsenoside Rg_3.



(III)



(IV)

Such acetylation reaction can be conducted using acetic anhydride(Ac_2O) or acetyl chloride as an acetylating agent. In this case, the acetylating agent can be used in a molar ratio of 1:1-4, preferably 1:1.2-2 with respect to the compound (III) or (IV). It is appropriate to carry out this reaction at a temperature of -40°C to 20°C, preferably -40°C to 0°C, for 1 to 48 hours.

The novel saponin compound of formula (I) or (II) thus obtained can be further purified by a conventional working-up method, for example, selective crystallization, column chromatograpy, etc.

The novel saponin compounds (I) and (II) prepared according to the process of the present invention as mentioned above, have a potent anti-tumor activity, and therefore can be effectively used as an agent for prevention or treatment of cancerous disease such as hepatoma, gastric cancer, leukemia, etc. Therefore, the present invention also relates to an anti-tumor composition comprising as an active ingredient the compound (I) or (II) or the mixture thereof.

When the composition containing the compounds (I) and/or (II) of the present invention is applied for clinical purposes as an anti-tumor agent, it can be combined with pharmaceutically acceptable carriers to prepare various formulations conventionally used in the pharmaceu-

tical field, for example, oral preparations such as tablets, capsules, troches, solutions, suspensions, etc.; injectable preparations such as injectable solutions or suspensions, or ready-to-use dried powder which can be applied after re-constituted with injectable distilled water before injection, etc.; or locally applicable preparations such as ointments, creams, solutions, etc.

The carriers which can be used in the composition of the present invention are conventional ones in the pharmaceutical field, for example, binders, lubricants, disintegrating agents, excipients, solubilizers, dispersing agents, stabilizers, suspending agents, coloring agents, flavors and the like in the case of oral preparations; preservatives, agents for painlessness, solubilizers, stabilizers and the like in the case of injectable preparations; bases, excipients, lubricants, preservatives and the like in the case of locally applicable preparations. The pharmaceutical preparations thus produced can be administered orally, or parenterally such as for example intravenously, peritoneally, subcutaneously, or can be topically applied. In addition, the oral preparations may be administered together with an antacid or in the form of an enteric-coated preparation which is formulated by covering the orally administrable solid preparation such as tablet with the enteric coatings, in order to prevent decomposition of the preparation by gastric acid when it is administered per orally.

Although the administration dosage to a human being of the novel saponin compounds (I) and (II) according to the present invention can be selected depending on the absorption, inactivating rate and excretion rate of the active component in the body, age, sex and condition of the subject patient, severity of the disorders to be treated and the like, it is generally administered to an adult in an amount of 5 to 500mg, preferably 10 to 200mg daily.

Therefore, when the composition of the present invention is formulated into the dosage unit form, each of the dosage unit form can contain the compounds (I) and/or (II) in an amount of 5 to 500mg, preferably 10 to 200mg on the basis of the effective amount range as mentioned above. If necessary, the dosage unit form thus formulated can be administered using a specialized method according to the judgement of the specialist who arranges or observes the administration and the requirement of the individuals. The total daily dosage can also be divided into several portions and administered over several times, preferably 1 to 6 times.

The present invention is more specifically explained by the following examples and test examples. However, it should be understood that the present invention is not limited to those examples in any manner.

EXAMPLE 1 : Preparation of ginseng extract containing the compounds (I) and (II)

100g of fresh ginseng was introduced into a sealed container and then heated for 2 hours at 130°C. The obtained processed ginseng was extracted with 200ml of methanol to obtain the methanol extract and then methanol was removed from the extract by evaporation. The remaining residue was suspended in 100ml of water, extracted 3 times with 100ml of ether, and then the remaining aqueous layer was extracted 3 times with 100ml of butanol saturated with water to obtain the butanol extract containing saponins. This butanol extract was dried and then subjected to silica gel column chromatography(eluent; ethyl acetate/methanol/ water = 20:1:1). According to the result of TLC analysis (developing solvent: ethyl acetate/methanol/ water = 10:1:1) of the eluates, 30mg of a fraction containing 50% of the desired compound (I) having the Rf value of 0.25 and 25mg of a fraction con-

taining 55% of the compound (II) having the Rf value of 0.27 were obtained, respectively.

EXAMPLE 2 : Preparation of ginseng extract containing the compounds (I) and (II)

10kg of dried root-hair ginseng was extracted by refluxing with 20L of methanol for 4 hours. The ginseng extract thus obtained was dried under reduced pressure. The resulting ginseng extract in the form of a syrup was introduced into an autoclave and then heated for 4 hours at 120°C. The heat-treated ginseng extract was subjected to silica gel column chromatography according to the same method as Example 1 and then the eluates were applied for TLC analysis (developing solvent: ethyl acetate/methanol/water = 10:1: 1) to obtain 3g of a fraction containing 50% of the desired compound (I) having the Rf value of 0.25 and 2g of a fraction containing 60% of the compound (II) having the Rf value of 0.27, respectively.

Example 3 : Preparation of the compound (I)

1g of the fraction containing the compound (I) prepared in Example 2 above was subjected to silica gel column chromatograpy according to the same manner as Example 1 using the mixed solvent of ethyl acetate/methanol/water(20:1:1) as an eluent to obtain 400mg of a fraction containing 92% of the desired compound (I). The fraction thus obtained was crystallized from the solvent mixture of methanol/water (1:1, v/v) to obtain 200mg of the desired compound (I).

The compound (I) thus obtained exhibits the following physico-chemical characteristics :

Chemical name : 3ß,12ß,20ß-trihydroxy-damar-24-ene-3-O-ß-

D-6"-O-acetyl-glucopyranosyl-(1→2)-ß-D-glucopyranoside

Mass spectrum (FAB$^+$, m/z) : 827([M+H]$^+$), 849([M+Na]$^+$)
(FAB$^-$, M/Z) : 825([M-H]$^-$)

CNMR(δ ppm, pyridine-d5) : 16.1, 16.2, 16.7, 17.1, 17.4, 18.3, 20.9, 22.4, 22.7, 25.5, 26.3, 26.8, 27.7, 30.8, 30.9, 34.8, 35.4, 36.7, 39.1, 39.4, 39.8, 49.2, 50.1, 50.2, 51.6, 56.2, 62.5, 64.5, 70.7, 70.8, 71.1, 73.6, 75.0, 75.1, 77.6, 77.7, 78.2, 83.9, 88.9, 104.6, 105.8, 125.8, 130.5, 171.0

Example 4 : Preparation of the compound (II)

1g of the fraction containing the compound (II) prepared in Example 2 above was subjected to silica gel column chromatography according to the same manner as Example 1 using the solvent mixture of ethyl acetate/methanol/water(20:1:1) as an eluent to obtain 300mg of a fraction containing 95% of the desired compound (II). The fraction thus obtained was crystallized from the solvent mixture of methanol/water (1:1, v/v) to obtain 150mg of the desired compound (II).

The compound (II) thus obtained exhibits the following physico-chemical characteristics :

Chemical name : 3ß,12ß-dihydroxy-damar-20(22),24-diene-3-O-ß-D-6"-O-acetyl-glucopyranosyl-(1→2)-ß-D-glucopyranoside

Mass spectrum (FAB$^+$, m/z) : 809([M+H]$^+$), 831([M+Na]$^+$)

CNMR(δ ppm, pyridine-d5) : 13.1, 15.7, 15.9, 15.9, 17.1, 17.8, 18.5, 20.9, 25.7, 26.8, 27.1, 27.4, 28.1, 32.2, 32.6, 35.4, 37.1, 39.3, 39.8, 40.3, 50.8,

50.9, 51.1, 51.2, 56.5, 62.9, 64.8, 70.9, 71.1, 71.5, 75.4, 77.9, 78.1, 78.1, 78.6, 84.3, 89.2, 104.9, 106.2, 123.5, 125.2, 131.0, 140.8, 171.0

Example 5 : Synthesis of the compound (I)

50mg of ginsenoside Rg_3 was dried under reduced pressure and 1ml of 2,4,6-collidine was added thereto and then stirred for 10 minutes at -40°C. 10μl of acetyl chloride was introduced thereinto and then the mixture was allowed to react for 3 hours. The reaction mixture was warmed slowly to room temperature and allowed to stand for 1 hour at room temperature. 1ml of methanol was added and the reaction solution was subjected to silica gel column chromatography according to the same manner as Example 1 to obtain 20mg of the desired compound (I).

Example 6 : Synthesis of compound (II)

50mg of $\Delta^{20(22)}$-ginsenoside Rg_3 was dried under reduced pressure and 1ml of 2,4,6-collidine was added thereto and then stirred for 10 minutes at -40°C. 10μl of acetyl chloride was introduced thereinto and then the mixture was allowed to react for 3 hours. The reaction mixture was warmed slowly to room temperature and allowed to stand for 1 hour at room temperature. 1ml of methanol was added and the reaction solution was subjected to silica gel column chromatography according to the same manner as Example 1 to obtain 25mg of the desired compound (II).

Test Example 1 : Anti-tumor activity of the compounds (I) and (II)

The anti-tumor activity of the novel saponin compounds of formulas (I) and (II) according to the present invention was determined by the method for measuring the incor-

poration amount of ^{3}H thymidine as described in the following.

13.8g of DMEM (Dulbecco's Modified Eagle's Medium, manufactured by Gibco Co.) was dissolved in 1L of deionized water and then adjusted to pH 7.4 with sodium carbonate and hydrochloric acid solution. Then, 10% calf serum, 1×10^{-7}M of insulin and 50mg/L of gentamycin were added thereto. The mixture was then sterilized by means of a millipore filter to prepare the culture solution. To this culture solution was inoculated human hepatoma sk-Hep-1 cell line, which was distributed from Cancer Research Center of Seoul National University in Korea, in a ratio of 1×10^{6} cells per 25cm^{2} of the T flask area, which was then incubated for 48 hours in an incubator of 37°C while keeping 5% CO_2 gas. The culture product was transferred to a 24-well incubator and subcultured for one day, and then each of the compounds (I) and (II) dissolved in 70% ethanol was added thereto to a concentration of 0.01 to 10μM, respectively. The same volume of 70% ethanol, instead of compounds (I) and (II), was added to the control group. 12 hours after treatment with each of the compounds (I) and (II), ^{3}H-labelled thymidine was added to a concentration of 1μCi/ml. After 12 hours, the medium was removed from each well and the cells were fixed with methanol, washed with PBS and then washed twice with 10% trichloroacetic acid to remove the unreacted radioactive thymidine. The cells were dissolved in 1N sodium hydroxide solution and neutralized with 1N hydrochloric acid and then the radioactivity introduced into DNA was measured by scintillation counter(Pharmacia 1024). The measured results are described in the following Table 1.

Table 1. Incorporation amount of radioactive thymidine into the DNA of hepatoma sk-Hep-1 cells depending on the concentration of compounds (I) and (II).

	Compound (I)		Compound (II)	
Concentration (μM)	dpm/well (Mean ± S.E.)	Percentage to control	dpm/well (Mean ± S.E.)	Percentage to control
Control	17812 ± 819	100.0	25828 ± 1918	100.0
0.01	23281 ± 1256	130.7	19242 ± 570	74.5
0.1	15016 ± 1231	84.3	15658 ± 1873	60.6
0.5	15665 ± 921	87.9	15137 ± 1467	58.6
1	15729 ± 302	88.3	13646 ± 2127	52.8
2.5	13155 ± 563	73.9	6421 ± 1527	24.9
5	733 ± 145	4.1	430 ± 105	1.7
10	728 ± 254	4.1	457 ± 115	1.8

Note S.E. : Standard error

As can be seen from the results described in Table 1 above, both the compounds (I) and (II) remarkably decrease the amount of incorporated radioactive thymidine at a concentration of 0.1μM or more, particularly 5μM or more. Therefore, it can be seen that the compounds (I) and (II) significantly inhibit the growth of hepatoma sk-Hep-1 cells.

Test Example 2 : Cell growth-inhibitory activity of the compounds (I) and (II) against human hepatoma cells

13.8g of DMEM (Dulbecco's Modified Eagle's Medium, manufactured by Gibco Co.) was dissolved in 1L of deionized water and then adjusted to pH 7.4 with sodium carbon-

ate and hydrochloric acid solution. Then, 10% calf serum, $1x10^{-7}$M of insulin and 50mg/L of gentamycin were added thereto. The mixture was then sterilized by means of a millipore filter to prepare the culture solution. To this culture solution was inoculated human hepatoma cell line sk-Hep-1, which was distributed from Cancer Research Center of Seoul National University, in a ratio of $1x10^6$ cells per $25cm^2$ of the T flask area, which was then incubated for 48 hours in an incubator of 37°C while keeping 5% of CO_2 gas. The culture product was transferred to a 96-well incubator in a concentration of 10^3 cells per well and subcultured for one day, and then each of the compounds (I) and (II) dissolved in 70% ethanol was added thereto to a concentration of 0.1 to 50μM, respectively. After 24 and 48 hours from the treatment with each of the compounds (I) and (II), 20μl of 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) dissolved in PBS(phosphate buffered saline) (5mg/ml) was added thereto and treated for 4 hours at 37°C to produce the insoluble formazane. The reaction mixture was centrifuged and the supernatant was removed. Then, 100μl of DMSO(dimethylsulfoxide) was added to dissolve the formazane precipitate and then the optical density at 570nm was measured by means of automatic plate reader as an index of the amount of formazane thus produced. The measured results are described in the following Tables 2 (after 24 hours) and 3 (after 48 hours).

Table 2. MTT analysis against human hepatoma sk-Hep-1 cells depending on the concentration of compounds (I) and (II) (after 24 hours)

	Compound (I)		Compound (II)	
Concentration (μM)	O.D. (Mean ± S.D.)	Percentage to control	O.D. (Mean ± S.D.)	Percentage to control
Control	0.98 ± 0.03	100.0	0.94 ± 0.16	100.0
0.01	0.88 ± 0.05	89.7	0.70 ± 0.02	74.6
0.1	0.74 ± 0.01	76.0	0.65 ± 0.00	69.5
0.5	0.68 ± 0.05	69.6	0.59 ± 0.04	62.7
1	0.61 ± 0.03	61.9	0.52 ± 0.03	55.9
5	0.50 ± 0.01	50.7	0.46 ± 0.04	48.7
10	0.42 ± 0.04	43.1	0.36 ± 0.01	38.2
25	0.17 ± 0.03	17.3	0.24 ± 0.03	26.0

Table 3. MTT analysis against human hepatoma sk-Hep-1 cells depending on the concentration of compounds (I) and (II) (after 48 hours)

	Compound (I)		Compound (II)	
Concentration (μM)	O.D. (Mean ± S.D.)	Percentage to control	O.D. (Mean ± S.D.)	Percentage to control
Control	1.02 ± 0.02	100.0	1.07 ± 0.04	100.0
0.01	0.89 ± 0.04	87.8	0.90 ± 0.04	83.8
0.1	0.70 ± 0.03	69.0	0.69 ± 0.03	64.1
0.5	0.42 ± 0.02	41.6	0.42 ± 0.01	38.7
1	0.34 ± 0.02	33.0	0.36 ± 0.01	33.8
5	0.18 ± 0.01	18.1	0.28 ± 0.04	26.0

Table 3. (continued)

	Compound (I)		Compound (II)	
Concentration (μM)	O.D. (Mean ± S.D.)	Percentage to control	O.D. (Mean ± S.D.)	Percentage to control
10	0.14 ± 0.01	13.7	0.14 ± 0.01	12.7
25	0.12 ± 0.01	11.6	0.12 ± 0.01	11.0

Note O.D. : Optical density
S.D. : Standard deviation

From the results described in Tables 2 and 3 above, it can be seen that since the optical density(O.D.) decreases accordingly as the concentration of the compound (I) or (II) increases, the compound of the present invention significantly inhibits the cell-growth of hepatoma sk-Hep-1 cells.

Test Example 3 : Acute toxicity test of the compounds (I) and (II)

40 mice weighing 20 to 40g were used as test animal and divided into 2 groups including 20 mice, respectively. Each of the compounds (I) and (II) according to the present invention was suspended in 1ml of physiological saline and orally administered to each group. After 14 days from administration, the number of survived test animal was counted. To the control group, 1ml of physiological saline was orally administered. The results are described in the following Table 4.

Table 4. Acute toxicity of the compounds (I) and (II) against mouse

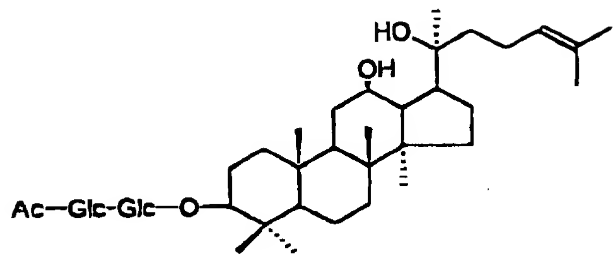
Test group	Dosage (mg/kg, Oral)	Number of Test animals	Number of Survived animals
A	1000	20	20
B	1000	20	20

Note A : Compound (I) receiving group
B : Compound (II) receiving group

From the results described in Table 4 above, it can be seen that the novel ginseng saponin compounds (I) and (II) according to the present invention have no substantial toxicities.

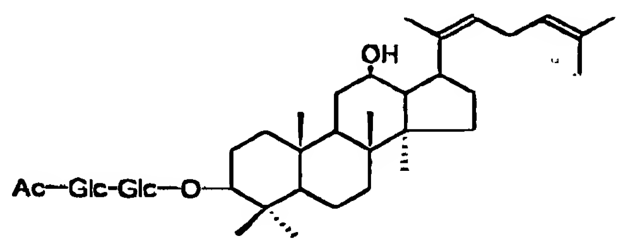
WHAT IS CLAIMED IS :

1. A ginseng saponin compound having the following formula (I):



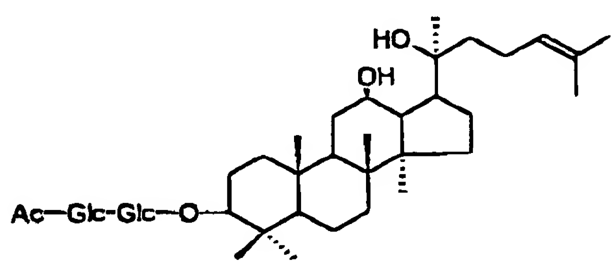
(I)

2. A ginseng saponin compound having the following formula (II) wherein the configuration of $\Delta^{20(22)}$ is zusammen or entgegen:



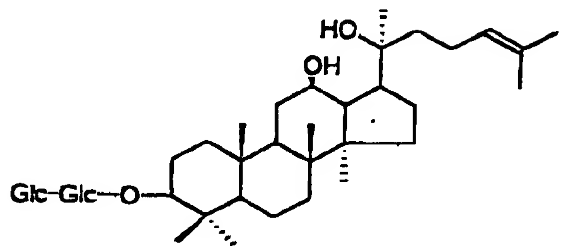
(II)

3. A process for preparing a compound having the following formula (I):



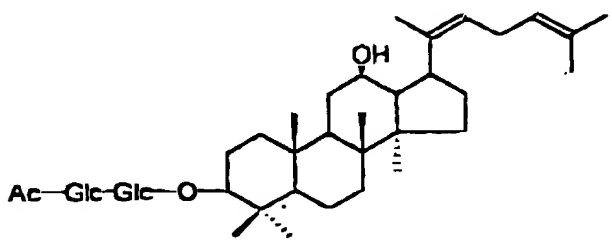
(I)

which comprises acetylating ginsenoside Rg_3 having the following formula (III):



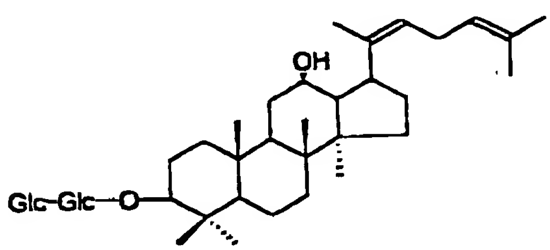
(III)

4. A process for preparing a compound having the following formula (II):



(II)

which comprises acetylating $\Delta^{20(22)}$-ginsenoside Rg_3 having the following formula (IV):



(IV)

5. A process for preparing the compounds of formulas (I) and (II) as defined in claims 1 and 2, characterized in that an extract from the plant of Panax genus with

water or lower alcohol is heated for 0.5 to 20 hours at a temperature of 110 to 180°C, the processed ginseng thus obtained is extracted with water, an organic solvent or a mixture thereof and then the extract is concentrated under reduced pressure, suspended in water and then extracted with a nonpolar organic solvent, the aqueous layer is separated and extracted with a polar organic solvent, and the obtained extract is subjected to chromatography to obtain a fraction containing the said compounds (I) and (II), which is then crystallized from a solvent mixture of water and lower alcohol.

6. An anti-tumor composition comprising the compound of formula (I) as defined in claim 1, the compound of formula (II) as defined in claim 2 or a mixture thereof as an active component, together with a pharmaceutically acceptable carrier.

7. The composition of claim 6 which is formulated into a pharmaceutical dosage unit form.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 96/00123

A. CLASSIFICATION OF SUBJECT MATTER

IPC^6: C 07 J 17/00, 75/00; A 61 K 31/705

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC^6: C 07 J 17/00, 75/00; A 61 K 31/705

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

–

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

QUESTEL: G-DARC

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Chemical Abstracts, Vol.112, No. 19, 07 May 1990 (Columbus, Ohio, USA), page 432, abstract No.175597h, I.KITAGAWA et al., "Chemical studies on crude drug processing. VI. Chemical structures of malonyl-ginsenosides Rb_1, Rb_2, Rc and Rd isolated from the root of Panax ginseng C.A. Meyer", & Chem.Pharm.Bull. 1989, 37(11) 2961-70.	1.5
A	Chemical Abstracts, Vol.123, No.9, 28 August 1995 (Columbus, Ohio, USA), page 748, abstract No. 122844g, D.S.KIM et al., "Preparation and structur determination of a new glycoside, (20E)-ginsenoside Rh_3, and its isomer from diol-type ginseng saponins.", & Yakhak Hoechi 1995, 39(1) 85-93.	2,5
A	Chemical Abstracts, Vol.107, No.3, 20 July 1987 (Columbus, Ohio, USA), page 642, abstract No. 23596r, L.N.ATOPKINA et al., "Glycosylation of dammarane type triterpenoids. IV. β-D-Glucopyranosides of betulafolienetriol and its derivatives", & Khim. Prir. Soedin. 1968, (3), 301-12.	1

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 January 1997 (16.01.97)	24 January 1997 (24.01.97)

Name and mailing address of the ISA/ AT	Authorized officer
AUSTRIAN PATENT OFFICE Kohlmarkt 8-10 A-1014 Vienna Facsimile No. 1/53424/535	Hofbauer Telephone No. 1/53424/225

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 96/00123

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DE 40 01 895 A1 (HARRIER GMBH) 25 July 1991 (25.07.91), pages 1-7.	2,6,7

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/KR 96/00123

Im Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
DE A1 4001895	25-07-91	AU A1 71489/91	21-08-91
		CA AA 2073692	24-07-91
		CN A 1053239	24-07-91
		EP A1 594581	04-05-94
		HU A0 9202121	28-12-92
		HU A2 61778	01-03-93
		IL A0 96982	29-03-92
		IL A1 96982	31-01-96
		JP T2 5504565	15-07-93
		JP A2 8109192	30-04-96
		US A 5278296	11-01-94
		US A 5496806	05-03-96
		WO A1 9111452	08-08-91
		AU A1 24011/92	23-02-93
		CA AA 2113819	04-02-93
		CN A 1073179	16-06-93
		CN B 1031643	24-04-96
		EP A1 597018	18-05-94
		EP A4 597018	07-02-96
		IL A0 102566	14-01-93
		JP T2 6501499	17-02-94
		WO A1 9301821	04-02-93

Form PCT/ISA/210 (patent family annex) (July 1992)

THIS IS EXHIBIT " C " REFERRED TO IN THE

AFFIDAVIT OF F. GEOFFREY HERRING

MADE BEFORE ME THIS

2nd day of December, 2002

A Commissioner for taking Affidavits
or a Notary Public

Metabolism f 20(*S*)- and 20(*R*)-Ginsenoside R_{g3} by Human Intestinal Bacteria and Its Relation to *in Vitro* Biol gical Activities

Eun-Ah BAE,[a] Myung Joo HAN,[a] Min-Kyung CHOO,[b] Sun-Young PARK,[b] and Dong-Hyun KIM*,[b]

Department of Food and Nutrition,[a] and College of Pharmacy,[b] Kyung Hee University, 1, Hoegi, Dongdaemunku, Seoul 130–701, Korea. Received June 6, 2001; accepted August 23, 2001

When ginsenoside R_{g3} was anaerobically incubated with human fecal microflora, all specimens metabolized ginsenoside R_{g3} to ginsenoside R_{h2} and protopanaxadiol. The main metabolite was ginsenoside R_{h2}. 20(*S*)-ginsenoside R_{g3} was quickly transformed to 20(*S*)-ginsenoside R_{h2} or 20(*S*)-protopanaxadiol in an amount 19-fold that compared with the transformation of 20(*R*)-ginsenoside R_{g3} to 20(*R*)-ginsenoside R_{h2} or 20(*R*)-protopanaxadiol. Among the bacteria isolated from human fecal microflora, *Bacteroides* sp., *Eubacterium* sp., and *Bifidobacterium* sp. metabolized ginsenoside R_{g3} to protopanaxadiol *via* ginsenoside R_{h2}. However, *Fusobacterium* sp. metabolized ginsenoside R_{g3} to ginsenoside R_{h2} alone. Among ginsenoside R_{g3} and its metabolites, 20(*S*)-protopanaxadiol and 20(*S*)-ginsenoside R_{h2} exhibited the most potent cytotoxicity against tumor cell lines, 20(*S*)- and 20(*R*)-protopanaxadiols potently inhibited the growth of *Helicobacter pylori*, and 20(*S*)-ginsenoside R_{h2} inhibited H^+/K^+ ATPase of rat stomach.

Key words ginsenoside R_{g3}; intestinal bacteria; ginsenoside R_{h2}; protopanaxadiol; cytotoxicity; *Helicobacter pylori*

Most herbal medicines are orally administered and their components inevitably come into contact with intestinal microflora in the alimentary tract. These components may be transformed before they are absorbed from the gastrointestinal tract. Studies on the metabolism of herbal medicine components by human intestinal microflora are therefore of great importance in understanding their biological effects.[1,2)]

Among herbal medicines, ginseng (the root of *Panax ginseng* C.A. MEYER, Araliaceae) is frequently used in Asian countries as a traditional medicine. The major components of ginseng are ginsenosides.[3,4)] These ginsenosides have been reported to show various biological activities including anti-inflammatory activity[5)] and antitumor effects.[6,7)] The pharmacological actions of these ginsenosides has been explained by the biotransformation of ginsenosides by human intestinal bacteria.[8—12)] Transformed 20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol (IH-901, compound K) from ginsenosides R_{b1}, R_{b2} and R_c induces an antimetastatic or anticarcinogenic effect.[13—15)] In addition, ginsenosides R_{b1}, R_{b2}, and R_c are transformed to ginsenoside R_{g3} by treatment with mild acid such as stomach acid.[16)] Furthermore, ginsenoside R_{g3} is a main component of Red Ginseng.[17)] However, studies on the metabolism of ginsenoside R_{g3} by human intestinal bacteria are not complete.

Therefore we investigated the human intestinal bacteria capable of metabolizing ginsenoside R_{g3} and its related biological activities, such as *in vitro* cytotoxicity and anti-*Helicobacter pylori* (HP) activity.

MATERIALS AND METHODS

Materials and Bacterial Strains Sodium thioglycolate and ascorbic acid were purchased from Sigma Chemical Co. (U.S.A.). General anaerobic medium (GAM) was purchased from Nissui Pharmaceutical Co., Ltd., (Japan). Tryptic soy broth was purchased from Difco Co. (U.S.A.). *p*-Nitrophenyl β-D-glucopyranoside (PNG) was purchased from Sigma (U.S.A.). The other chemicals were of analytical reagent grade.

Tumor cell lines were purchased from the Korean Cell Bank. HP ATCC43504 was purchased from ATCC, HP NCTC11638 was purchased from NCTC. HP82516 and HP4 clinically isolated from Korean gastroscopic samples were used. They were inoculated onto brucella agar plates supplemented with 7% horse serum and cultured for 3 days at 37 °C under microaerophilic conditions (AnaeroPak Campylo: 85% N_2, 10% CO_2, and 5% O_2).

Isolation of Transformants of Ginsenoside R_{b1} under Mild Acidic Conditions Ginsenoside R_{b1} (2 g) was treated in mild acidic conditions[16)] at 37 °C for 2 h, as previously reported, concentrated at 60 °C and extracted with *n*-BuOH. From this BuOH fraction, 20(*S*)- and 20(*R*)-ginsenoside R_{g3} were isolated according to the previous method.[18)] The BuOH fraction was chromatographed on a silica gel column using $CHCl_3$–MeOH–H_2O (10 : 3 : 1, lower layer) to produce Δ^{20}-ginsenoside R_{g3} (0.05 g), and *isomeric* 20(*S*)- and 20(*R*)-ginsenoside R_{g3} (0.6 g). The *isomeric* mixture was dissolved in pyridine, and acetic anhydride was added drop by drop in ice bath and stirred at room temperature for 10 h. The reaction mixture was dispersed in ice water and extracted three times with EtOAc. The organic layer was washed with 5% HCl, saturated aqueous $NaHCO_3$, and brine, followed by drying over anhydrous magnesium sulfate. The residue was applied to silica gel column chromatography and eluted with dichloromethane–EtOAc (8 : 1) to afford peracetylated-20(*S*)-ginsenoside R_{g3} and peracetylated-20(*R*)-ginsenoside R_{g3}. These compounds were each dissolved in 5% NaOH/*n*-BuOH (20 ml) in an ice bath and stirred at room temperature overnight. After the reaction mixtures were washed with water and evaporated to dryness, the residues were applied to silica gel column chromatography using $CHCl_3$–MeOH–H_2O (9 : 3 : 1, v/v) to give 20(*S*)-ginsenoside R_{g3} (0.21 g) and 20(*R*)-ginsenoside R_{g3} (0.12 g), respectively.

20(*S*)-Ginsenoside R_{g3} (**4**): Colorless needles, mp 248—250 °C (dec.), FAB-MS (*m/z*) 786 $[M+1]^+$.

20(*R*)-Ginsenoside R_{g3} (**5**): Colorless needles, mp 299—302 °C (dec.), FAB-MS (*m/z*) 786 $[M+1]^+$.

Is lation of Metabolites of Ginsenoside R_{g3} by Human Intestinal Bacteria The reaction mixture containing 100 mg of 20(*S*)-ginsenoside R_{g3} (or 20(*R*)-ginsenoside R_{g3}) and

* To whom correspondence should be addressed. e-mail: dhkim@khu.ac.kr

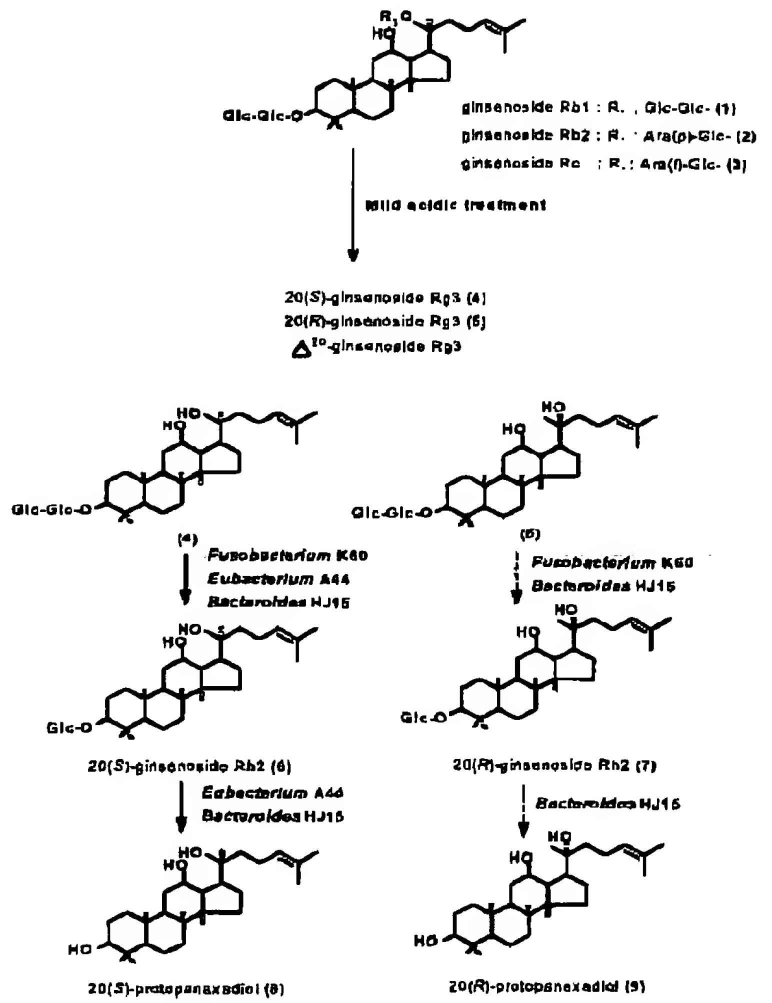


Chart 1. Proposed Metabolic Pathway of Ginsenoside R_{g3} by Human Intestinal Bacteria

Each metabolic pathway was potently catalyzed by the bacteria listed from bacteria tested in this experiment. →, main pathway; ⇢, minor pathway.

500 mg fresh fecal suspension was incubated for 24 h (or 72 h for 20(*R*)-ginsenoside R_{g3}) at 37 °C. The reaction mixture was adjusted to pH 2 with HCl, extracted with ethylacetate, evaporated to dryness, and applied to silica gel column chromatography; solvent, $CHCl_3$–MeOH–H_2O (65 : 35 : 10 v/v, lower phase). 20(*S*)-ginsenoside R_{h2} (12 mg) and 20(*R*)-ginsenoside R_{h2} (20 mg), and 20(*S*)-protopanaxadiol (15 mg) and 20(*R*)-protopanaxadiol (5 mg) were isolated.

Ten grams of fresh feces were suspended with 100 ml of anaerobic diluted media and centrifuged at 200×*g* for 5 min, and the precipitate was discarded. The supernatant was centrifuged at 10000×*g* for 20 min. The precipitate was washed twice with saline and used in the experiment.

20(*S*)-Ginsenoside R_{h2} (**6**): Colorless needles, mp 219—221 °C (dec.), FAB-MS (*m/z*) 623 $[M+1]^+$.

20(*R*)-Ginsenoside R_{h2} (**7**): Colorless crystals, mp 208—210 °C (dec.), FAB-MS (*m/z*) 623 $[M+1]^+$.

20(*S*)-protopanaxadiol (**8**): Colorless needles, mp 198—200 °C (dec.), Electron-impact (EI)-MS (*m/z*) 459 $[M]^+$.

20(*R*)-protopanaxadiol (**9**): White needless, mp 236—238 °C (dec.), EI-MS (*m/z*) 459 $[M]^+$.

Screening of Bacteria Metabolizing Ginsenoside R_{g3} Among human feces tested, potent ginsenoside R_{g3}-hydrolyzing fresh feces were anaerobically diluted 10^3- to 10^7-fold. Two hundred microliters of the diluted fecal suspension were inoculated on GAM agar plates. The plates were anaerobically incubated at 37 °C for 24 h. More than 100 colonies isolated from several plates, or bacteria previously isolated from human intestinal bacteria (*Bacteroides* HJ-15, *Bacteroides* JY-6, *Eubacterium* A-44, *Bifidobacterium* K-111, and *Fusobacterium* K-60)[13] were cultured in 50 ml of tryptic soy broth containing 0.01% sodium thioglycolate and 0.1% ascorbic acid (TSTA), and then each cultured cell was centrifuged at 3000×*g* for 10 min and washed twice with saline. The activities of these collected cells in metabolizing ginsenoside R_{g3} were measured using the assay method described.

Assay f Metabolic Activity of Ginsenoside R_{g3} by Human Intestinal Bacteria The reaction mixture containing 100 µl of 1 mM ginsenoside R_{g3} and 100 µl of fecal sus-

pension (or bacterial suspension cultured in TSTA broth) was incubated for 12 h at 37 °C. The reaction mixture was adjusted to pH 2 with HCl, extracted with ethylacetate, evaporated, and assayed by TLC: TLC plates, Silica gel $60F_{254}$ (Merck Co., U.S.A.); developing solvent, $CHCl_3$–MeOH–H_2O (65 : 35 : 10 v/v, lower phase). The plates were stained by spraying with MeOH–H_2SO_4 (95 : 5 v/v), followed by heating. The stained TLC plates were then analyzed using a TLC scanner (Shimadzu model CS-9301PC, Japan).

Each isolated bacterium was cultured in 50 ml of TSTA broth and centrifuged at 3000×*g* for 30 min. Each collected bacterial pellet was suspended in 50 mM phosphate buffer and used as a crude enzyme solution.

Assay of β-Glucosidase Activity β-Glucosidase enzyme activity was assayed according to our previous method.[19]

Time Course of the Metabolism of Ginsenoside R_{g3} by Human Fecal Microflora Ginsenoside R_{g3}-metabolizing activity was measured as follows. Two milliliters of fresh human fecal suspension (250 mg/ml) or the isolated intestinal bacterial suspension (250 mg/ml or 1 g/ml) were added to 8 ml of anaerobic diluted medium[11] containing 0.3 mM 20(*S*)-ginsenoside R_{g3} (or 20[*R*]-ginsenoside R_{g3}) and then was incubated at 37 °C for 1 d, and an aliquot (0.5 ml) of the reaction mixture was periodically extracted twice with 1 ml of ethylacetate. The ethylacetate fraction was analyzed by TLC. Ginsenoside R_{g3} and its metabolites were identified and assayed against authentic compounds isolated according to our previous method.

The fresh feces of a healthy volunteer (2 g) were collected, suspended in 48 ml of anaerobic dilution medium, centrifuged at 200×*g* and the supernatant was centrifuged at 10000×*g* for 30 min.

The isolated bacteria were cultured in 500 ml of TSTA broth and centrifuged at 10000×*g* for 30 min and washed with the anaerobic dilution medium. The fecal and bacterial precipitates (250 mg) were resuspended in 1.75 ml of anaerobic dilution medium.

Assay of Anti-HP Activity A growth inhibition assay of HP was performed according to the previous method.[19]

Preparation and Assay of HP Urease The preparation of partially purified urease from HP was performed according to our previous method.[19] Urease activity was determined by the indophenol method.[20] Acetohydroxamic acid was used as a positive control.

Preparation and Assay of Rat Stomach H^+/K^+-ATPase Gastric H^+/K^+-ATPase was partially purified from the parietal cell-rich fraction of male Sprague-Dawley rat (200—250 g) stomach as described by Saccomani and Mukidjam.[21]

Rat gastric H^+/K^+-ATPase activity was also determined according to the modified method of Saccomani and Mukidjam.[21]

***In Vitro* Cytotoxicity Assay** The *in vitro* cytotoxicity was tested against L1210 (mouse lymphocytic leukemia cell line), P388 (mouse lymphoid neoplasma cell line), A549 (human lung carcimoma), and Me180 (human cervix uterine carcinoma) cell lines by MTT [3-(3,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay according to the method of Carmichael *et al.*[22]

RESULTS

Metabolism f Ginsenoside R_{g3} by Human Intestinal Microflora When ginsenoside R_{b1} decomposed under mild acidic conditions (0.1 N HCl), two additional spots were observed on TLC. They were tentatively designated as Δ^{20}-ginsenoside R_{g3} and isomeric ginsenoside R_{g3} in the order of increasing polarity. The isomeric ginsenoside was separated into 20(*S*)- and 20(*R*)-ginsenoside R_{g3} by acetylation and solubility. The acid-labile nature of ginsenosides was observed even if the concentration of HCl was as low as 0.01 N HCl. These results suggest that this reaction of ginsenoside R_{g3} could occur in the stomach or during the fermentation of ginseng.

Therefore, to investigate the metabolic process of ginsenside R_{g3} before absorption in the intestine, 20(*S*)- and 20(*R*)-ginsenside R_{g3} were incubated with human fecal suspension. 20(*S*)-Ginsenoside R_{h2} and 20(*S*)-protopanaxdiol, and 20(*R*)-protopanaxdiol and 20(*R*)-ginsenoside R_{h2} were observed as the metabolites, respectively. The main metabolites of these compounds were 20(*S*)- and 20(*R*)-ginsenoside R_{h2} during 24-h incubation, respectively.

When 20(*S*)- and 20(*R*)-ginsenoside R_{g3}-hydrolyzing activity was first assayed in fecal specimens from five different human subjects, their transforming activities were detected in all specimens. However, these activities varied depending on the individual samples. The mean of the activities transforming 20(*S*)- and 20(*R*)-ginsenoside R_{g3} to 20(*S*)- and 20(*R*)-ginsenoside R_{h2} were 0.57±0.20 and 0.03±0.002 nmol/h/mg wet weight of feces, respectively (Fig. 1). The metabolic activity of 20(*S*)-ginsenoside R_{g3} to 20(*S*)-ginsenoside R_{h2} was 19-fold higher than that of 20(*R*)-ginsenoside R_{g3} to 20(*R*)-ginsenoside R_{h2}. When 20(*S*)-ginsenoside R_{g3} was incubated with human fecal microflora, it began to be transformed to 20(*S*)-ginsenoside R_{h2}, which was transformed to 20(*S*)-protopanaxadiol (Fig. 2). However, 24 h after incubation the main metabolite was 20(*S*)-ginsenoside R_{h2}. The metabolic pathway of 20(*R*)-ginsenoside R_{g3} was similar to that of 20(*S*)-ginsenoside R_{g3}. However, its metabolism was weakly

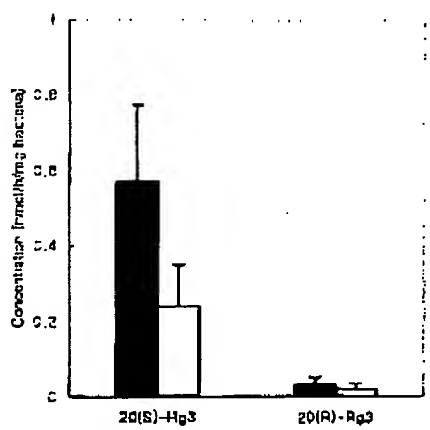


Fig. 1. The Activity Transforming Ginsenoside R_{g3} to Ginsenoside R_{h2} and Protopanaxadiol by Human Fecal Microflora

Human fecal suspension was prepared and its activity was assayed as described in Materials and Methods. Transforming activity of ginsenoside R_{g3} to ginsenoside R_{h2} was calculated from total transformed ginsenoside R_{h2} and protopanaxadiol. Symbols indicate the following: ■, activity transforming ginsenoside R_{g3} to ginsenoside R_{h2}; □, activity transforming ginsenoside R_{g3} to protopanaxadiol.

processed.

β-Glucosidase Activities of Human Intestinal Bacteria We cultured more than 30 bacteria isolated from human feces and measured their β-glucosidase activities to evaluate the glycoside-metabolizing activity of the bacteria isolated from human feces (Table 1). Using PNG as a substrate, most bacteria produced potent β-glucosidase activity. These bacteria were also capable of hydrolyzing 20(*S*)-ginsenoside R_{g3} mainly giving 20(*S*)-ginsenoside R_{h2}. The most potently 20(*S*)-ginsenoside R_{g3}-hydrolyzing bacteria were *Bacteroides* HJ-15, *Bifidobacterium* K-111, *Eubacterium* A-44, and *Fusobacterium* K-60. These bacteria, except for *Fusobacterium* K-60, transformed 20(*S*)-ginsenoside R_{g3} to 20(*S*)-protopanaxadiol *via* 20(*S*)-ginsenoside R_{h2} (Fig. 3). However, 20(*R*)-ginsenoside R_{g3} was hydrolyzed very little by most intestinal bacteria except for *Bacteroides* HJ-15 and *Fusobacterium* K-60. Nevertheless, these ginsenoside R_{g3}-transforming bacteria also hydrolyzed PNG, a synthetic substrate of β-glucosidase, but PNG-hydrolyzing activity was not proportional to ginsenoside R_{g3}-hydrolyzing activity.

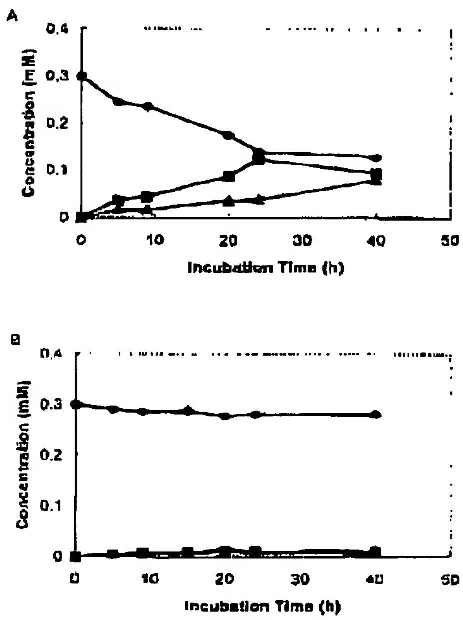


Fig. 2. Time Course of 20(*S*)- and 20(*R*)-Ginsenoside R_{g3} Transformation by Human Fecal Microflora

Human fecal suspension was prepared and their metabolites were assayed as described in Materials and Methods. A, 20(*S*)-ginsenoside R_{g3}; B, 20(*R*)-ginsenoside R_{g3}. ◆, 20(*S*)- or 20(*R*)-ginsenoside R_{g3}; ■, 20(*S*)- or 20(*R*)-ginsenoside R_{h2}; ▲, 20(*S*)- or 20(*R*)-protopanaxadiol.

Biological Activities of Ginsenoside R_{g3} and Its Metabolites The inhibitory effect of ginsenoside R_{g3} and its metabolites on the growth of HP was measured (Table 2). Ginsenosides R_{b1}, R_{g3}, and R_{h2} did not inhibit HP growth. However, 20(*S*)- and 20(*R*)-protopanaxadiols inhibited HP growth at MICs of 50—100 μg/ml. The inhibitory effects of these compounds on the activity of HP urease and H^+/K^+ ATPase were also measured (Table 3). Most of the tested compounds did not inhibit these enzymes. However, 20(*S*)-ginsenosides R_{g3} and R_{h2} weakly inhibited H^+/K^+ ATPase of the rat stomach, with their IC_{50} values of 0.6 and 0.48 mg/ml, respectively.

We also investigated the *in vitro* cytotoxic activity of ginsenoside R_{g3} and its metabolites on the tumor cell lines (Table 4). Ginsenoside R_{b1} and 20(*R*)-ginsenoside R_{g3} did not exhibit cytotoxicity against the tumor cell lines. 20(*S*)-Ginsenoside R_{g3} only exhibited weak cytotoxicity. However, the metabolites of 20(*S*)-ginsenoside R_{g3}, 20(*S*)-ginsenoside R_{h2} and 20(*S*)-protopanaxadiol, showed potent cytotoxicity against tumor cell lines, with IC_{50} values of 22—33 and 18—33 μM, respectively.

DISCUSSION

The main components of ginseng, which is frequently used in Asia, are ginsenoside R_{b1} and R_{b2}. These ginsenosides are likely transformed to 20-β-*O*-glucopyranosyl-20(*S*)-protopanaxadiol (IH-901) or 20(*S*)-protopanaxadiol *via* ginsenoside R_d or gypenoside XVII by human intestinal bacteria.[11,13,23]

Han *et al.* reported that ginsenoside R_{b1} and R_{b2} were transformed to ginsenoside R_{g3} when these compounds were incubated in mildly acidic conditions,[16] and suggested that this transformation of ginsenosides R_{b1} and R_{b2} to ginsenoside R_{g3} could occur in the stomach. In addition, ginsenoside R_{g3} is a major component of Red Ginseng rather than of Ginseng.[17] When ginsenoside R_{g3} was incubated with human fecal microflora, it was transformed to ginsenoside R_{h2} and protopanaxadiol. When 20(*S*)-ginsenoside R_{g3} was incubated

Table 1. β-Glucosidase Activity of Representative Intestinal Bacteria Isolated from Human Feces

	PNG (nmol/(min·mg))	Transforming activity[a] (nmol/(h·mg))			
		20(*S*)-Ginsenoside R_{g3}		20(*R*)-Ginsenoside R_{g3}	
		20(*S*)-R_{h2}	20(*S*)-Ppd	20(*R*)-R_{h2}	20(*R*)-Ppd
Bacteroides HJ15	1.191	1.455	0.002	0.06	0.01
Bacteroides JY6	0.242	0.06	0.01	<0.01	<0.01
Bifidobacterium K-111	0.620	0.102	0.010	0	0
Escherichia coli HGU-3	0.022	0	0	0	0
Eubacterium A44	0.451	0.371	0.149	<0.01	<0.01
Eubacterium L8	0.104	0.106	<0.01	0	0
Fusobacterium K-60	0.249	0.759	0	0.02	0
Streptococcus S2	0.100	0.039	0	0	0
Streptococcus S10	0.129	0.032	<0.01	<0.01	<0.01

a) Transforming activity of ginsenoside R_{g3} to ginsenoside R_{h2} was calculated from total transformed ginsenoside R_{h2} and protopanaxadiol.

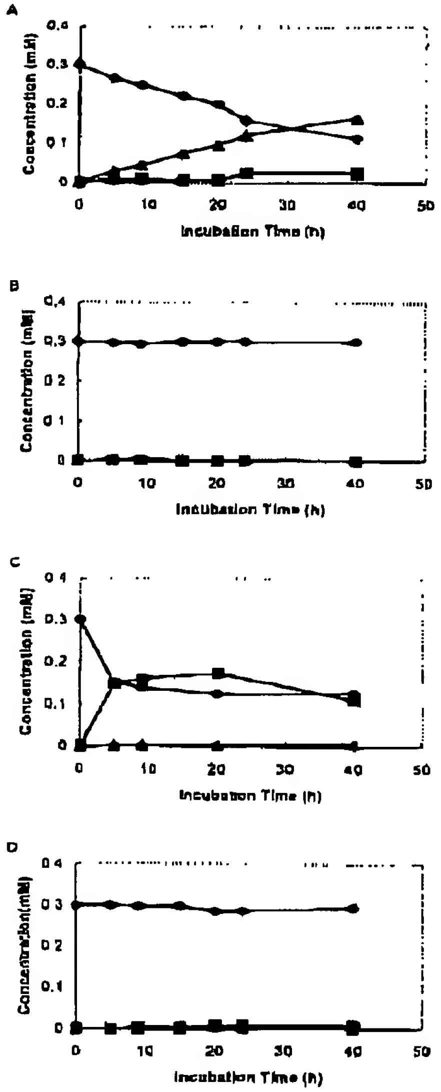


Fig. 3. Time Course of 20(*S*)- and 20(*R*)-Ginsenoside R_{g3} Transformation by Human Intestinal Bacteria *Eubacterium* A-44 and *Fusobacterium* K-60

The intestinal bacterial suspension was prepared and their metabolites were assayed as described in Materials and Methods. A, 20(*S*)-Ginsenoside R_{g3} was treated with *Eubacterium* A-44 (1 g); B, 20(*R*)-Ginsenoside R_{g3} was treated with *Eubacterium* A-44 (1 g); C, 20(*S*)-Ginsenoside R_{g3} was treated with *Fusobacterium* K-60 (250 mg); D, 20(*R*)-Ginsenoside R_{g3} was treated with *Fusobacterium* K-60 (250 mg). ●, 20(*S*)- or 20(*R*)-ginsenoside R_{g3}; ■, 20(*S*)- or 20(*R*)-ginsenoside R_{h2}; ▲, 20(*S*)- or 20(*R*)-protopanaxadiol.

with intestinal bacterial strains previously isolated, it was mainly transformed to ginsenoside R_{h2}. However, 20(*S*)-ginsenoside R_{g3} was potently metabolized to 20(*S*)-ginsenoside R_{h2} and 20(*S*)-protopanaxadiol, while 20(*R*)-ginsenoside R_{g3} was barely metabolized by the human fecal microflora and intestinal bacterial strains. Most of the isolated intestinal bacteria hydrolyzed PNG. However, some intestinal bacteria only could convert ginsenoside R_{g3} to protopanaxadiol *via* ginsenoside R_{h2}. This suggests that 20(*S*)-ginsenoside R_{g3} is not only a good substrate of β-glucosidase of intestinal bacteria, but is also highly s luble in water compared with 20(*R*)-ginsenoside R_{g3}.

Table 2. Anti-*Helicobacter pylori* Activity of 20(*S*)- and 20(*R*)-Ginsenoside R_{g3} and Their Metabolites

Compound	MIC (μg/ml)			
	HP ATCC43504	HP NCTC11638	HP 82516	HP 4
Ginsenoside R_{b1}	>100	>100	>100	>100
20(*S*)-Ginsenoside R_{g3}	>100	>100	>100	>100
20(*R*)-Ginsenoside R_{g3}	>100	>100	>100	>100
20(*S*)-Ginsenoside R_{h2}	>100	>100	>100	>100
20(*R*)-Ginsenoside R_{h2}	>100	>100	>100	>100
20(*S*)-Protopanaxadiol	50	50	50	50
20(*R*)-Protopanaxadiol	50	50	100	100

Table 3. Inhibitory Effects of Ginsenoside R_{g3} and Its Metabolites on HP Urease and Rat Stomach H^+/K^+ ATPase

Compound	IC_{50} (mg/ml)	
	HP urease	Stomach H^+/K^+ ATPase
Ginsenoside R_{b1}	>1	>1
20(*S*)-Ginsenoside R_{g3}	>1	0.6
20(*R*)-Ginsenoside R_{g3}	>1	>1
20(*S*)-Ginsenoside R_{h2}	>1	0.48
20(*R*)-Ginsenoside R_{h2}	>1	>1
20(*S*)-Protopanaxadiol	>1	>1
20(*R*)-Protopanaxadiol	>1	>1
Acetohydroxamic acid	0.18	—
Omeprazole	—	0.21

Table 4. Cytotoxicity of 20(*S*)- and 20(*R*)-Ginsenoside R_{g3} and Their Metabolites against Tumor Cell Lines

Compound	IC_{50} (μM)			
	L1210	P388	A549	Mc180
Ginsenoside R_{b1}	>100	>100	>100	>100
20(*S*)-Ginsenoside R_{g3}	47	58	>100	>100
20(*R*)-Ginsenoside R_{g3}	>100	>100	>100	>100
20(*S*)-Ginsenoside R_{h2}	22	33	31	28
20(*R*)-Ginsenoside R_{h2}	>100	>100	>100	>100
20(*S*)-Protopanaxadiol	18	33	28	28
20(*R*)-Protopanaxadiol	>100	>100	>100	>100

Based on these findings, we suggest the following metabolic pathway of ginsenosides R_{b1} and R_{b2} (Chart 1). If ginsenosides R_{b1} and R_{b2} are orally administered, they could be transformed to ginsenoside R_{g3} in the stomach. This ginsenoside R_{g3}, which is in Red Ginseng as well as metabolized from ginsenosides R_{b1} and R_{b2} in the stomach, should be metabolized to ginsenoside R_{h2} or 20(*S*)-protopanaxadiol in the human intestine. However, if orally administered ginsenosides R_{b1} and R_{b2} are not transformed to ginsenoside R_{g3} in the stomach, they should be metabolized to IH 901 or 20(*S*)-protopanaxadiol in the human intestine.

To understand what the active compounds of ginsenosides and ginseng extract are when they are orally administered in humans, we measured s me biological activities, anti-HP, H^+/K^+ ATPase-inhibitory, and cytotoxic activity against tumor cell lines of ginsenoside R_{g3} and its metabolites. We

found that the cytotoxicity of ginsenosides against tumor cell lines was increased when 20(*S*)-ginsenoside R_{g3} was metabolized to 20(*S*)-ginsenoside R_{h2} or 20(*S*)-protopanaxdiol by human intestinal microflora. Anti-HP activity was also increased when ginsenoside R_{g3} was metabolized to protopanaxadiol. Furthermore, ginsenoside R_{h2} inhibited H^+/K^+ ATPase more potently than ginsenoside R_{g3}. These results suggest that the natural glycosides ginsenosides R_{b1} and R_{g3} are prodrugs, which can be transformed to active compounds by intestinal microflora. Finally, we believe that 20(*S*)-ginsenoside R_{h2} and 20(*S*)-protopanaxadiol transformed from ginseng saponins could play an important role in the antitumor activity, and that protopanaxadiol contained in ginseng extract could inhibit HP growth, while ginsenoside R_{g3} cannot be transformed to protopanaxadiol in the stomach.

Acknowledgement This work was supported by The National Agricultural Cooperative Federation Grant (2001).

REFERENCES

1) Kobashi K., Akao T., *Bioscience Microflora*, **16**, 1—7 (1997).
2) Kim D.-H., Yu K.-W., Bae E.-A., Park H.-J., Choi J.-W., *Biol. Pharm. Bull.*, **21**, 360—365 (1998).
3) Nagai M., Sanada S., Tanaka N., Tanaka O., Shibata S., *Chem. Pharm. Bull.*, **20**, 1212—1216 (1972).
4) Karikura M., Miyaze T., Tanizawa H., Taniyama T., Takino Y., *Chem. Pharm. Bull.*, **38**, 2357—2361 (1991).
5) Wu J. Y., Gardner B. H., Murphy C. I., Seals J. R., Kensil C. R., Recchia J., Beltz G. A., Newman G. W., Newman M. J., *J. Immunol.*, **148**, 1519—1525 (1992).
6) Sato K., Mochizuki M., Saiki I., Yoo Y. C., Samukawa K., Azuma I., *Biol. Pharm. Bull.*, **17**, 635—639 (1994).
7) Mochizuki M., Yoo Y. C., Matsuzawa K., Sato K., Saiki I., Tono-oka S., Samukawa K., Azuma I., *Biol. Pharm. Bull.*, **18**, 1197—1202 (1995).
8) Karikura M., Miyase T., Tanizawa H., Taniyama T., Takino Y., *Chem. Pharm. Bull.*, **39**, 400—404 (1991).
9) Kanaoka M., Kato H., Shimada F., Yano S., *Chem. Pharm. Bull.*, **40**, 314—317 (1992).
10) Kanaoka M., Akao T., Kobashi K., *J. Trad. Med.*, **11**, 241—245 (1994).
11) Akao T., Kanaoka M., Kobashi K., *Biol. Pharm. Bull.*, **21**, 245—249 (1998).
12) Akao T., Kida H., Kanaoka M., Hattori M., Kobashi K., *J. Pharm. Pharmacol.*, **50**, 1155—1160 (1998).
13) Bae E.-A., Park S.-Y., Kim D.-H., *Biol. Pharm. Bull.*, **23**, 1481—1485 (2000).
14) Wakabayashi C., Hasegawa H., Murata J., Saiki I., *Oncol. Res.*, **9**, 411—417 (1998).
15) Lee S. J., Sung J. H., Lee S. J., Moon C. K., Lee B. H., *Cancer Lett.*, **144**, 39—43 (1999).
16) Han B. H., Park M. H., Han Y. N., Woo W. S., Sankawa U., Yahara S., Tanaka O., *Planta Medica*, **44**, 146—149 (1982).
17) Kaku T., Kawashima Y., *Arzneim. Forsch. Drug Res.*, **30**, 936—940 (1980)
18) Back N. I., Kim D. S., Lee Y. H., Park J. D., Lee C. B., Kim S. I., *Arch. Pharm. Res.*, **18**, 164—168 (1995).
19) Bae E.-A., Han M. J., Kim D.-H., *Planta Medica*, **65**, 442—443 (1999).
20) Odake S., Morikawa T., Tsuchiya M., Imamura L., Kobashi K., *Biol. Pharm. Bull.*, **17**, 1329—1332 (1994).
21) Saccomani G., Mukidjam E., *Biochim. Biophys. Acta*, **912**, 63—73 (1987).
22) Carmichael J., DeGraff W. G., Gazdar A. F., Minna J. D., Mitchell J. B., *Cancer Res.*, **47**, 936—940 (1987).
23) Hasegawa H., Sung J.-W., Benno Y., *Planta Medica*, **63**, 436—440 (1997).